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Processes for the preparation of fibrinogen

The present invention relates to processes for the
5   purification of fibrinogen, and to readily solubilised
fibrinogen preparations.

Fibrinogen is a blood plasma protein which is
concerned in clot formation. It is converted into
fibrin monomer by the action of the plasma protease
10   thrombin. Fibrin monomers cluster together to form a
weak clot and are then cross-linked by the action of
activated factor XIII (i.e. factor XIIIa) to a form a
stronger clot. Fibrinogen is used in therapy in
combination with thrombin in the so-called fibrin
15   sealants to achieve haemostasis, to seal wounds and for
the controlled adhesion of tissue. Fibrinogen
concentrates are also used for replacement therapy
treatment of patients with fibrinogen deficiency
(afibrinogenaemia) which may be inherited or acquired.
20   For all clinical applications, it is important to
have highly pure fibrinogen in order to minimise any
undesirable side effects resulting from, for example,
the presence of unwanted contaminating proteins. In
particular, it is desirable for fibrinogen preparations
25   for clinical use to be free of plasminogen and plasmin
(Blomback B., Blomback M., "Purification of human and
bovine fibrinogen", Arkiv for Kemi 1956; 10:415-443, and
Mosesson M.W., "The preparation of human fibrinogen free
of plasminogen", Biochim Biophys Acta 1962, 57:204-213).
30   Plasminogen is the inactive precursor of plasmin, a
fibrinolytic enzyme which digests fibrin clots.
Therefore the presence of plasminogen in a fibrinogen
preparation intended for use in vivo is undesirable
because any plasmin generated from the plasminogen at
35   the site of clot formation may then destabilise the
clot.

Plasminogen tends to co-purify with fibrinogen and

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its removal can be difficult. Some clinical fibrinogen
preparations therefore contain anti-fibrinolytic agents
to inhibit any plasmin or plasminogen present (e.g.
aprotinin, a bovine protein inhibitor of plasmin; or

5   tranexamic acid, a synthetic plasmin inhibitor also
associated with neurotoxic side-effects). One advantage
of separating plasminogen from fibrinogen is that there
is then no need to use such fibrinolytic inhibitors in
the clinical fibrinogen preparation.

10   In addition, it is highly desirable that fibrinogen
derived from human or animal sources is treated to
inactivate any blood-borne viruses which may be present,
for example hepatitis virus or HIV. Various methods of
virus inactivation are known in the art, including

15   pasteurisation, dry heat treatment and solvent-detergent
treatment (Pathogen Inactivation of Labile Blood
Products, Council of Europe Expert Committee and Blood
Transfusion Study Group on Pathogen Inactivation in
Labile Blood Products, *Transfusion Medicine*, 2001, **11**,

20   149-175).

           Dry heat treatment is known to be effective for the
inactivation of both enveloped and some non-enveloped
viruses, whilst solvent-detergent treatment is known to
be effective for the inactivation of enveloped (i.e.

25   lipid coated) viruses such a hepatitis B.

           Various methods for the purification of fibrinogen
are known in the art. However, prior art purification
methods suffer from various disadvantages. For example,
precipitation methods do not allow easy incorporation of

30   a solvent-detergent (SD) virus inactivation step, as the
removal of SD reagents is much more efficiently effected
chromatographically. Chromatography methods may not
separate fibrinogen from plasminogen in a single step,
which may lead to the need for additional chromatography

35   to adsorb plasminogen, or the need to add an
anti-fibrinolytic agent to the final fibrinogen
preparation to combat residual plasminogen. In

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addition, not all the prior art methods are suitable for
the purification of fibrinogen from a wide range of
fibrinogen-containing solutions (including plasma and
recombinant fractions).

5   US 5,169,936 has previously suggested that
immobilised metal ion affinity chromatography (IMAC)
might be used in the preparation of human fibrinogen.
However, no examples of such a method are disclosed, nor
is there any suggestion that IMAC might be used for the
10   separation of fibrinogen from plasminogen.

         It is also known that the dissolution of fibrinogen
concentrates can be difficult, and often requires the
use of elevated temperatures or prolonged stirring (see
US 5,260,420 and EP-A 0804933). Due to the instability
15   of liquid solutions of fibrinogen over time, fibrinogen
preparations for clinical use are marketed either in the
form of a deep-frozen solution or as a lyophilisate
(i.e. a freeze dried preparation). Before use, the
commercial product must be either thawed or
20   reconstituted from the lyophilisate. Both these
measures require significant time and effort.

         It would therefore be advantageous to provide
alternative methods for the purification of fibrinogen,
in particular a method which is applicable to any
25   fibrinogen containing starting material and which allows
incorporation of one or more virus inactivation steps.
It would also be advantageous to provide a method for
the separation of fibrinogen from plasminogen.
Furthermore, it would be advantageous to provide a
30   lyophilised, and preferably heat treated, fibrinogen
concentrate which can be readily redissolved at room
temperature.

         In one aspect, the present invention therefore
provides a method for the separation and purification of
35   fibrinogen and at least one other protein which
comprises the steps of:

         (a) loading a solution comprising fibrinogen and at

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least one other protein onto an immobilised metal ion
affinity chromatography matrix under conditions such
that the fibrinogen and the at least one other protein
both bind to the matrix, and

5   (b) selectively eluting the fibrinogen and the at
least one other protein separately from the matrix. The
fibrinogen and the at least one other protein may be
collected separately and each processed further as
required.

10   Preferably, the solution comprising fibrinogen is a
fibrinogen-containing plasma fraction. Preferably, the
at least one other protein is plasminogen.

In a further aspect, the present invention provides
a method for the separation of fibrinogen from

15   plasminogen comprising use of immobilised metal ion
affinity chromatography. Preferably, the method
comprises the steps of:

(a) loading a solution comprising fibrinogen and
plasminogen onto an immobilised metal ion affinity

20   chromatography matrix under conditions such that at
least the fibrinogen binds to the matrix, and

(b) selectively eluting the fibrinogen from the
matrix. Preferably the plasminogen also binds to the
matrix, and the plasminogen and the fibrinogen may be

25   selectively eluted separately from the matrix.

As used herein, references to the separation and/or
purification of fibrinogen include the joint separation
and/or co-purification of fibrinogen and factor XIII
together from starting materials comprising both

30   fibrinogen and factor XIII.

The starting material for the methods of the
invention may be any fibrinogen-containing solution,
including human or animal plasma or a plasma fraction,
cell culture fractions from recombinant technology,

35   fractions derived from milk from transgenic animals,
etc. Preferred starting materials are plasma fractions
such as cryoprecipitate, heparin precipitate and cold

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precipitate. More preferred starting materials include
heparin precipitate and cryoprecipitate. Other
preferred starting materials include those further
comprising plasminogen and/or factor XIII.

5   The starting material may be prepared by any
suitable method known in the art, including via gene
manipulation, for example in cell culture or transgenic
species. For example, cryoprecipitate may be prepared
according to the method of Gunson H.H., Bidwell E., Lane
10   R.S., Wensley R.T., Snape T.J., "Variables involved in
cryoprecipitate production and their effect on Factor
VIII activity", British Journal of Haematology,
1978;43:287 - 295; heparin precipitate may be prepared
according to the method of Winkelman L., Owen N.E.,
15   Evans D.R., Evans H.E., Haddon M.E., Smith J.K., Prince
P.J., Williams J.D., Lane R.S., "Severely heated
therapeutic Factor VIII concentrate of high specific
activity", Vox Sanguinis, 1989;57:97 - 103; and cold
precipitate according to the method of Smith J.K., Evans
20   D.R., Stone V., Snape T.J., "A Factor VIII concentrate
of intermediate purity and higher potency", Transfusion,
1979;19:299 - 306.

          Unwanted contaminants in the starting material
which may be separated from the fibrinogen using the
25   methods of the invention may include other proteins (for
example plasma proteins such as plasminogen), reagents
from earlier processing steps (for example elements of
cell culture media or solvent-detergent reagents),
viruses and prions. It is particularly preferred that
30   plasminogen be removed, so that the addition of plasmin
inhibitors (anti-fibrinolytic agents) to the fibrinogen
may be avoided.

          The fibrinogen-containing solution is loaded onto
an IMAC matrix. Preferably, the matrix is present in a
35   column for ease of processing. Any suitable metal ion
may be used, for example copper, zinc or nickel,
preferably copper. Suitable immobilised metal ion

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affinity chromatography gels for use in the process of
the invention include methacrylate gel with
multi-substituted ligands on the side chain spacers
(e.g. Fractogel EMD Chelate from Merck), methacrylate
gel with single chelating groups on the spacer arm (e.g.
Toyopearl Chelate from Tosoh Biosep) and cross-linked
agarose gel (e.g., chelating Sepharose FF from Amersham
Biosciences). A preferred gel is Toyopearl AF chelate
650(M) from Tosoh Biosep.

The loading conditions, including the buffer used,
should be chosen such that the fibrinogen, and any
factor XIII if present, in the starting material are
bound to the gel. Unwanted contaminants which do not
bind to the gel may then be removed by washing. For
example, if the starting material has previously been
subjected to a solvent-detergent inactivation step, any
solvent or detergent reagents remaining do not bind to
the gel and are easily removed by washing.
Alternatively, if the unwanted contaminants bind to the
gel they may be removed by selective elution before the
fibrinogen is eluted, or they may remain bound to the
gel whilst the fibrinogen is selectively eluted.
Additionally, washing the gel and bound protein(s) may
also help to remove any viruses which may be present in
the chromatography feedstocks.

It has been found that plasminogen binds less
tightly than fibrinogen or factor XIII to metal chelate
chromatography gels. Any plasminogen present in the
starting material may therefore be removed by washing,
by selective elution using a low concentration solution
of low molecular weight competitive chelating compound,
or by changing conditions so as to reduce the binding
strength, for instance by reducing the pH or ionic
strength, whilst the fibrinogen remains bound. Suitable
chelating compounds include amino acids, for example
alanine, leucine and lysine, imidazole, citrate salts
and ethylenediaminetetraacetic acid (EDTA). A preferred

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chelating compound for the elution of plasminogen is
alanine. The concentration of the chelating compound
should be chosen such that plasminogen is eluted whilst
the fibrinogen remains bound to the gel. The exact

5   concentration will depend on the eluant used. For
example, concentrations of < about 20mM should
selectively remove plasminogen in the presence of bound
fibrinogen. Suitable concentrations for the elution of
plasminogen include ≤ 20mM of alanine or leucine, ≤ 10mM

10   lysine and < 10mM imidazole.
     The fibrinogen may then be eluted using a higher
concentration of the same or a different chelating
compound, or by reducing the pH or ionic strength.
Preferred chelating compounds for the elution of

15   fibrinogen are amino acids, preferably lysine or
arginine, and imidazole. A more preferred eluant
comprises arginine. For example, the fibrinogen may be
eluted using a > 20mM solution of the chelating
compound. The conditions for the elution of the

20   fibrinogen (concentration and pH) should be chosen such
that the fibrinogen is removed from the gel but the
metal ion is not, in order to minimise contamination of
the product with metal ions.
     Removal of plasminogen is advantageous as the

25   fibrinogen may then be used clinically without the need
for addition of any anti-fibrinolytic to the clinical
preparation. A further advantageous feature is that the
plasminogen which has been separated from the fibrinogen
by IMAC may then be further processed to yield a

30   plasminogen concentrate for clinical use. IMAC may
therefore be used to prepare both plasminogen and
fibrinogen from a starting solution comprising both
plasminogen and fibrinogen.
     It is an advantageous feature of the processes of

35   the invention that any factor XIII in the starting
material tends to co-elute with the fibrinogen. The
presence of measurable (>1u/ml) factor XIII in the final

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fibrinogen preparations may be beneficial if the
fibrinogen is to be used clinically. It has been shown
that the concentration of factor XIII has an effect in
some *in vitro* tests of fibrin sealants, although there
5   is no evidence that factor XIII is required for clinical
efficacy, and fibrin sealant products with no measurable
factor XIII activity have been used with clinical
effect. When used in a blood environment (e.g. for
haemostasis), the patient's endogenous factor XIII will
10   be present to effect clot cross-linking. Where this is
not the case it is possible that the presence of factor
XIII in the product might be beneficial. Since factor
XIII is a catalytic enzyme it can operate effectively
even at low concentration.

15   The present invention therefore further provides a
method the co-purification of fibrinogen and factor XIII
comprising use of immobilised metal ion affinity
chromatography. Preferably, the method comprises the
steps of:

20   (a) loading a solution comprising fibrinogen and
factor XIII onto an immobilised metal ion affinity
chromatography matrix under conditions such that the
fibrinogen and the factor XIII both bind to the matrix,
and

25   (b) selectively co-eluting the fibrinogen and the
factor XIII from the matrix.

Optionally, the fibrinogen-containing starting
material may be subjected to a solvent-detergent virus
inactivation treatment prior to the immobilised metal
30   ion affinity chromatography. Solvent detergent virus
inactivation may be carried out using reagents and
methods known in the art (see for example US 4,481,189,
US 4,613,501 and US 4,540,573, all of which are hereby
incorporated by reference). Suitable solvents include
35   tri-n-butyl phosphate (TnBP) and ether, preferably TnBP.
Suitable detergents include polysorbate (Tween) 80,
polysorbate (Tween) 20 and Triton X-100. A preferred

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detergent is polysorbate 20 and a particularly preferred combination is polysorbate 20 and TnBP.

The fibrinogen-containing fraction may be stirred with solvent and detergent reagents at a temperature and
5   for a time sufficient to inactivate any enveloped viruses that may be present. For example, the solvent detergent treatment may be carried out for about 1 hour at 25°C.

The fibrinogen recovered from the chromatography
10   step may then be further processed in order to formulate it for pharmaceutical use. For example, it may be concentrated by ultrafiltration to a concentration of approximately 15-30 mg/ml, and/or subjected to a further pathogen reduction step, for example nanofiltration.

15   The concentrate may then be formulated by the addition of a combination of suitable stabilisers, for example an amino acid, a carbohydrate, a salt, and a detergent. Particularly preferably, the product is formulated without the addition of any anti-fibrinolytic
20   agents or stabilising proteins such as albumin. The formulated product may then be sterilised by filtration and lyophilised (freeze-dried) for long term storage. Optionally, the freeze-dried product may be subjected to a dry heat treatment in a further virus inactivation
25   step. For example, it may be heated to about 80°C for about 72 hours or about 100°C for about 24 hours.

The combination of the amino acid, salt, carbohydrate and detergent used to formulate the fibrinogen product assists in stabilising it across the
30   freeze-drying and terminal heat treatment step. It also facilitates the reconstitution of the freeze-dried product. In particular, the stabilisers help stabilise any factor XIII present in the product, which is known to be highly labile.

35   The lyophilised and heat treated product may be reconstituted with water at ambient temperature in less than 15 minutes, preferably less than 10 minutes and

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most preferably less than 5 minutes to provide a
solution of fibrinogen with a concentration of at least
about 60 mg/ml.

It is an advantageous feature of the process of the
invention that plasminogen is removed, thus avoiding the
need for the addition of anti-fibrinolytic agents to the
final fibrinogen preparation.

As a further feature of the invention there is
therefore provided a lyophilised, preferably heat-
treated, fibrinogen preparation comprising fibrinogen
prepared according to one of the methods of the
invention, a carbohydrate, a buffer, a salt, an amino
acid and a detergent, and optionally factor XIII, the
preparation being capable of dissolution in water at
ambient temperature in less than 15 minutes, preferably
less than 10 minutes and more preferably less than 5
minutes to give a fibrinogen solution. Preferably the
concentration of fibrinogen in the final solution is at
least about 60 mg/ml.

Without wishing to be bound by any theory, it is
believed that combination of the salt, the detergent,
the amino acid and the carbohydrate facilitate rapid
dissolution of the preparation. The carbohydrate is
also believed to help preserve any factor XIII activity
present. The buffer controls the pH of the formulation.
The use of a combination of a carbohydrate, a buffer, a
salt, an amino acid and a detergent in the preparation
also stabilises the fibrinogen, and any factor XIII
present, without the need to add any other stabilisers,
for example other proteins such as albumin. This is
advantageous as addition of other proteins may be a
source of viral or other contamination of the product.
The fibrinogen preparations of the invention are thus
free of stabilising proteins, in particular albumin.
Preferably, the fibrinogen preparations of the invention
are also free of anti-fibrinolytic agents.

Suitable amino acids include arginine, suitable

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carbohydrates include sucrose, trehalose and raffinose,
preferably sucrose, suitable buffers include citrate
salts (e.g. sodium citrate) and phosphate salts (e.g.
sodium phosphate), suitable salts include sodium

5   chloride and suitable detergents include polysorbate 20.
Preferably, the detergent used in the final
formulation is the same detergent used for any earlier
solvent detergent virus inactivation step, the amino
acid formulant is the same one used to elute the

10   fibrinogen from the metal chelate column, and the salt
and buffer components are the same ones used during used
during purification thereby avoiding the need to remove
trace amounts of these components from the product. It
is also desirable to minimise the exposure of the

15   product to a multiplicity of reagents during
manufacture, as each reagent used is a source of
possible contamination or unwanted modification of the
product. Thus it is preferable if the final product
formulants are reagents which have already been used

20   during processing.
Suitable concentrations of the various components
will depend on the exact nature and source of the
fibrinogen and may be determined using routine trial and
error experiments. Suitable concentration ranges before

25   freeze drying include:
carbohydrate (preferably sucrose): about 0.5-
2.5%w/w;
detergent (preferably polysorbate 20): about 0.1-
0.5%w/w;

30   salt (preferably sodium chloride): about 50-250mM,
preferably about 50mM;
amino acid (preferably arginine): about 50-120mM,
preferably about 110mM.
Sufficient buffer is added to control the pH as desired,

35   for example at about pH 7.5.
Preferred formulations comprise about 0.5-2.5%w/w
sucrose, about 0.1-0.5%w/w polysorbate 20, about 50-

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250mM, more preferably about 50mM, sodium chloride, and about 50-120mM arginine at about pH 7.5.

The fibrinogen preparations of the invention are prepared by forming a solution of the components and then lyophilising the solution. After lyophilisation, the dry preparation is preferably subjected to a terminal heat treatment step in order to inactivate enveloped and non-enveloped viruses. For example, it may be heated to about 80°C for about 72 hours, or to about 100°C for about 24 hours. Heat treatment is known to be able to denature proteins, which can cause aggregation and reduce the solubility of the heat treated product. The other ingredients in the preparations of the invention help to stabilise the fibrinogen, and any factor XIII present, during the heat treatment step.

A more detailed description of preferred embodiments of the invention is now given:

Cryoprecipitate recovery from plasma

Frozen human plasma may be conditioned at around -11°C, thawed to between -0.5 and 2°C and the resulting cryoprecipitate recovered by centrifugation. The cryoprecipitate may be washed at < 4°C and recovered by centrifugation. The cryoprecipitate may be stored frozen.

Precipitation

The cryoprecipitate may be thawed with buffer (i.e. redissolved) to recover the proteins contained therein. The fibrinogen, fibronectin and factor XIII may then be precipitated using a suitable chemical agent, for example heparin, polyethylene glycol (PEG) or ethanol, or by adjustment of temperature and pH. The precipitate is then recovered, for example by centrifugation. This

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precipitate may be stored frozen.

Resuspension of the precipitate

5   The heparin or other precipitate may then be resuspended
        using an appropriate buffer and mixing for a suitable
        time and at a suitable temperature. The resulting
        preparation may then be clarified, for example by depth
        filtration or centrifugation, prior to 0.45 μm
10   filtration or smaller to remove any aggregates which may
        be present and which might shield viruses from the
        solvent-detergent inactivation reagents.

Solvent detergent treatment

15

        Solvent and detergent may then be added to the filtrate,
        and the mixture stirred at a suitable temperature so as
        to inactivate enveloped viruses. The solvent is
        preferably tri-n-butyl phosphate (TnBP), whereas the
20   detergent can be polysorbate 20, polysorbate 80 or
        Triton X-100, preferably polysorbate 20.

Chromatography

25   The solvent detergent treated material may then be
        applied directly to a metal chelate chromatography
        column, where the metal ion can be copper or any other
        suitable ion. Buffer conditions and loading are such
        that the solvent and detergent components are not
30   retained by the adsorbent whereas fibrinogen is. Any
        plasminogen which is bound may then be selectively
        eluted using a low concentration of a low molecular
        weight chelating compound, for example alanine. The
        eluate wash fraction containing the plasminogen may then
35   be further processed to yield a concentrate of
        plasminogen which may be used clinically. The
        fibrinogen may then be eluted at high yield using a

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higher concentration of the same or another chelating
compound, for example arginine. Factor XIII is co-
eluted with the fibrinogen. Fibrinogen is generally
present in the eluate at concentrations of about 3-20
5   mg/ml.

The column elution may be monitored by any suitable
method, for example by UV absorbance at a wavelength of
280 nanometres. This gives a measure of the protein
10   concentration in the eluate and can be used to cue
fraction collection and/or buffer changes.

Concentration

15   Optionally, the fibrinogen eluate may be concentrated
using ultrafiltration to give final concentrations of
about 15-30 mg/ml, preferably about 20-25 mg/ml.

Formulation

20

The fibrinogen concentrate may be formulated by the
addition of a combination of an amino acid, a
carbohydrate, a buffer, a salt and a detergent. The
formulated product may then be sterilised by filtration
25   to 0.2 μm and filled. Such filtration may remove or
reduce viruses and other pathogens, for example the
causative agent of Transmissible Spongiform
Encephalopathies (TSE), currently believed to be prions.
The formulation buffer (and freeze-drying conditions)
30   are preferably chosen such that the fibrinogen plug may
be reconstituted in water at room temperature in less
than 10 minutes. A preferred formulation is 110 mM
arginine, 1.5% w/w sucrose, 0.1% w/w polysorbate 20, 50
mM NaCl, 10 mM trisodium citrate.

35

Freeze-drying and heat treatment

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The product is freeze dried and then optionally heat treated at elevated temperatures in order to inactivate enveloped and non-enveloped viruses.

5   Microbiological contamination during the process is minimised by suitable sanitization of the IMAC medium and by filtration of buffers to remove bacterial contamination (e.g. by use of 0.2μm filters).

10   The fibrinogen prepared using the process of the invention may be used clinically, either alone or in combination with thrombin in a fibrin sealant kit. The present invention therefore also provides fibrinogen obtained according to the process of the invention, for
15   use in therapy, and pharmaceutical kits comprising fibrinogen obtained according the process of the invention in combination with thrombin. Preferred are kits comprising fibrinogen prepared according to the process of the invention and thrombin prepared according
20   to the applicant's co-pending PCT application No. (unknown) entitled "Process for the preparation of thrombin" filed on 7 July 2003 claiming priority from UK patent application No. 0216002.6 filed on 10 July 2002, the disclosure of which is hereby incorporated by
25   reference.

The invention will be further illustrated with reference to the following non-limiting Examples.

30   Fibrinogen was measured using the following methods: Heat Precipitation Assay based on a method published by Desvignes, P. and Bonnet, P., "Direct Determination of Plasma Fibrinogen levels by Heat Precipitation.' A comparison of the Technique against Thrombin Clottable
35   Fibrinogen with Spectrophotometry and Radial Immune Diffusion", Clinica Chimica Acta, 110 (1981), 9-17. Clot Time assay based on the method of Clauss (Clauss,

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A., Gerinnungsphysiologische Schnellmethode zur
Bestimmung des Fibrinogens. Acta Haematol 1957; 17:234-
46). Total Clottable Assay based on a method by
Blomback and Blomback, Arkiv fur Chemi., 1956,Chapter
10, 415-443.
Factor XIII was measured by photometric determination
(standard - normal human plasma). The method was based
on the following references:
Fickenscher, K., Aab, A., Stüber, W., "A Photometric
Assay for Blood Coagulation Factor XIII", Thromb.
Haemostas. 65 (1991), 535-540 and Solleder, E., Demuth,
D., Pfeiffer, C., Bomhard, M., Mayer, J., Eller, T.,
Brauer, P., Keller, F., Grun, J., Fickenscher, K.,
Wagner, C., "Klinische Prufung eines neuen
photometrischen Tests zur Bestimmung der Factor XIII
Aktivitat im Plasma", Lab Med. 16 (1992), 48-53.

Plasminogen and factor XIII were measured by ELISA.

Example 1: Cryoprecipitate recovery from plasma

Plasma was stored at less than -30°C until use. The
required weight of plasma was then conditioned at -11°C
before stripping the packaging. The plasma pool was
then thawed at <2.5°C in order to recover the
fibrinogen, factor VIII, von Willebrand factor (vWF) and
fibronectin cryoprecipitate from the plasma. This
precipitate was recovered by centrifugation and stored
frozen.

Example 2: Precipitation

Cryoprecipitate prepared according to Example 1 was
resuspended in 20 mM Tris/HCl pH 6.7 to a ratio of
0.024x the net plasma pool weight and thawed by warming
to between 20 and 40°C for >20 minutes. The pH was then
adjusted to 6.55 with 0.1 M HCl. A stock heparin

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solution was then added to give a final concentration of
0.88 mg/ml and the resulting mixture stirred for >2
minutes. This resulted in the precipitation of
fibrinogen and fibronectin leaving factor VIII and vWF
5   in solution. The heparin precipitate was then recovered
by centrifugation. This precipitate may be stored
frozen.

Example 3: Resuspension of the precipitate

10

Heparin precipitate prepared according to Example 2 was
resuspended in 20 mM NaH_2PO_4/Na_2HPO_4, 10 mM trisodium
citrate, 0.5 M NaCl pH 6.0 in a ratio of 1 part
precipitate to 5 parts buffer. This suspension was then
15   warmed to 40°C and incubated with mixing for >1 hour.
The resuspended heparin precipitate was then clarified
by filtration through two depth filters (Cuno 05SP and
Cuno 30LA) and a membrane filter (Sartobran 0.65/0.45
μm) to ensure the removal of aggregates which may shield
20   viruses from the subsequent solvent detergent treatment.

Example 4: Solvent detergent treatment

A solvent detergent stock solution of 20% v/v
25   polysorbate 20 and 6% v/v TnBP was then added to the
filtrate to give a final concentration of 1% v/v
polysorbate 20 and 0.3% v/v TnBP. The resulting mixture
was then stirred for not less than 1 hour at room
temperature.
30

Example 5: Chromatography

The solvent detergent treated heparin precipitate
prepared according to Example 4 was loaded onto a copper
35   charged Toyopearl AF chelate 650(M) column which had
been pre-equilibrated with not less than 5 bed volumes
of buffer 1 (EW1: 20 mM NaH_2PO_4/Na_2HPO_4, 10 mM trisodium

citrate, 0.5 M NaCl pH 6.0). The column was then loaded
with 3 bed volumes of treated heparin precipitate. The
rate of loading was no greater than 77 cm/hr. The bed
was then washed with 18 bed volumes of buffer 1. The
bound plasminogen was washed off using 15 bed volumes of
buffer 2 (EW2: 20 mM Na_2HPO_4, 15 mM alanine, 0.5 M NaCl
pH 7.5). The eluted plasminogen may be further
processed to yield a concentrate which may be used
clinically. Buffer conditions were then adjusted using
5 bed volumes of buffer 3 (EW3: 10 mM trisodium citrate,
50 mM NaCl pH 7.0). The bound fibrinogen was then
eluted using sufficient bed volumes of buffer 4 (EW4: 50
mM arginine, 10 mM trisodium citrate, 50 mM NaCl pH 7.5)
such that the A280nm (UV absorbance at a wavelength of
280 nm) returned to baseline. The copper ions were then
stripped from the resin using 5 bed volumes of 20 mM
Na_2HPO_4, 0.25 M NaCl, 50 mM EDTA pH 7.0.

Example 6: Concentration

The eluted fibrinogen prepared according to Example 5
was concentrated using a 100 kDa molecular weight cut
off membrane (Sartocon Sartorius). The membrane was
pre-washed using 50 mM arginine, 10 mM trisodium
citrate, 50 mM NaCl pH 7.5. The target concentration of
fibrinogen was 22 mg/ml.

Example 7: Formulation

The concentrated fibrinogen solution prepared according
to Example 6 was formulated by the addition of 710 mM
arginine, 16.5% w/w sucrose, 1.1% w/w polysorbate 20, 50
mM NaCl, 10 mM trisodium citrate pH 7.5 at a ratio of
1:10. The final formulation concentrations were 110 mM
arginine, 1.5% w/w sucrose, 0.1 % w/w polysorbate 20, 50
mM NaCl, 10 mM trisodium citrate. The formulated
product was then filtered to 0.2 μm (Sartobran 0.45/0.2

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μm, Sartorius).

Example 8: Freeze-drying and heat treatment

5   The product of Example 7 was aseptically filled into
     glass vials at 15 ml per vial and then freeze dried,
     stoppered and over sealed. The vials were heat treated
     at 80°C for not less than 72 hours in order to
     inactivate non-enveloped and enveloped viruses.

10

Example 9: Pilot scale chromatography of fibrinogen concentrate

     1274 g of heparin precipitate (prepared according to
15   Example 2) was re-suspended in a 5 fold volume of 20 mM
     NaH_2PO_4/Na_2HPO_4, 10 mM trisodium citrate, 0.5 M NaCl pH
     6.0 at 40°C in a water bath with constant stirring for
     greater than 1 hour. The resultant solution was
     filtered to 0.45 μm with the Cuno 05SP, Cuno 30LA and
20   Sartobran P 0.65/0.45 μm filter train. The solvent
     detergent mixture was then added to give final
     concentrations of 1% v/v polysorbate 20 and 0.3% v/v
     TnBP. The mixture was then stirred at room temperature
     for 1 hour.

25

     6391 g of solvent detergent treated heparin precipitate
     was loaded onto 2123 mL of Toyopearl AF Chelate 650(M)
     resin packed into a Amicon Vantage 130 column to give a
     settled height of 16 cm. The chromatography column was
30   sanitised using 5 bed volumes of 0.5 M NaOH and
     equilibrated with approximately 5 bed volumes of buffer
     1 (EW1, 20 mM NaH_2PO_4/Na_2HPO_4, 10 mM trisodium citrate,
     0.5 M NaCl pH 6.0). This was washed through with
     approximately 5 bed volumes of distilled water. The
·35 resin was then charged with metal ions using
     approximately 5 bed volumes of a 3 mg/ml copper sulphate
     solution. Loosely bound metal ions were removed using

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approximately 5 bed volumes of 50 mM arginine, 10 mM
trisodium citrate, 50 mM NaCl pH 7.5 and then
approximately 10 bed volumes of buffer 1. The load was
then applied, and the bed then washed with
approximately 18 bed volumes of buffer 1. The bound
plasminogen was washed off using approximately 16 bed
volumes of buffer 2 (EW2: 20 mM NaH_2PO_4/Na_2HPO_4, 15 mM
alanine, 0.5 M NaCl pH 7.5). Buffer conditions were
then adjusted using approximately 5 bed volumes of
buffer 3 (EW3: 10 mM trisodium citrate, 50 mM NaCl pH
7.0). The bound fibrinogen was then eluted using
approximately 5 bed volumes of buffer 4 (EW4: 50 mM
arginine, 10 mM trisodium citrate, 50 mM NaCl pH 7.5).
The copper ions were then stripped from the resin using
approximately 5 bed volumes of 20 mM Na_2HPO_4, 0.25 M
NaCl, 50 mM EDTA pH 7.0.

The concentrations and recovery data for fibrinogen,
plasminogen and Factor XIII are given in Table 1. The
clearance of TnBP and polysorbate 20 are given in Table
2.

Table 1

Sample	Volume ml	Fibrinogen		Factor XIII		Plasminogen	
		mg/ml	%	U/ml	%	μg/ml	%
Load	6391	11.81	(100)	0.8	(100)	43.9	(100)
FT	6407	<1	<8.49			0.01	0.02
EW1	31971	<1	<42.36	0.01	6.25	0.03	0.34
EW2	33800	<1	<44.78	0.05	33.06	7.74	93.25

EW3	10674	<1	<14.14	0.05	10.44	0.20	0.76
EP1	680						
EP2	8943	6.45	76.43	0.32	55.98	4.00	12.75
EP3	2060						
EDTA	9155	<1	<12.13			0.01	0.03

Abbreviations used: FT = flowthrough; EW1 =
equilibration wash 1; EW2 = equilibration wash 2; EW3 =
equilibration wash 3; EP1 = elution peak 1 (leading edge
of elution peak); EP2 = elution peak 2 (main elution
peak); EP3 = elution peak 3 (trailing edge of the
elution peak).

Table 2

Sample	Volume (ml)	TnBP		Polysorbate 20	
		Conc. (mg/l)	Recovery (%)	Conc. (mg/l)	Recovery (%)
Load	6391	2900	(100)	13700	(100)
EP2	8943	6.9	0.3	38	0.4

Abbreviations used: EP2 = elution peak 2 (main elution
peak).

The results showed that the three proteins, fibrinogen,
plasminogen and factor XIII, were efficiently captured
on the Cu^{2+} loaded Toyopearl and then 93% of the loaded
plasminogen was selectively removed by washing with the
15mM alanine buffer (EW2). The eluted fibrinogen
product contained 76% and 56% of the applied fibrinogen
and factor XIII respectively. The clearance of solvent
detergent chemicals was also efficient: only 0.3 and

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0.4% of the applied TnBP and polysorbate 20, respectively, were left in the product after chromatography.

5   Example 10: pilot scale ultrafiltration of fibrinogen

Two polysulphone Sartorius slice cassettes (0.1 m² each) were assembled into a Sartorius membrane holder and flushed with 7 1 deionised water. The assembly was
10   sanitised with a 1 1 flush of 1 M NaOH warmed to 40°C and then 5 1 of NaOH, recirculated for 1 hour. The system was then flushed with 10 1 deionised water at a cross flow velocity of 880 ml/min. The membrane was prepared with 5 1 of EW4 (50 mM arginine, 10 mM
15   trisodium citrate, 50 mM NaCl pH 7.5) with no applied back-pressure at the same cross-flow velocity.

8960 ml of eluted fibrinogen fraction was applied at an initial fibrinogen concentration of 5.24 mg/ml at a
20   maximum inlet pressure and trans-membrane pressure of 1.4 and 0.7 bar (140,000 and 70,000 Pa) respectively. The ultrafiltration took 1 hour 36 minutes and gave a retentate of 1227 ml and 23.8 mg/ml fibrinogen. The average flux was 28.8 L/m²/h and predicted gelling
25   concentrations was 31.0 mg/ml.

Example 11: choice of IMAC gels for purification of Fibrinogen

30   Several metal chelate chromatography gels were tested for their ability to bind and subsequently release fibrinogen. These are described in Table 3 below:

Table 3

35

Gel	Manufacturer	Base Matrix	Chelating group

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Toyopearl Chelate AF	Tosoh Biosep	Methacrylate	iminodiacetic acid
Toyopearl Chelate AF (modified spacer arm)	Tosoh Biosep	Methacrylate	iminodiacetic acid
Fractogel EMD Chelate	Merck	Methacrylate	iminodiacetic acid
Chelating Sepharose FF	Amersham Biosciences	Cross-linked agarose	iminodiacetic acid

The starting material was cryoprecipitate which contained fibrinogen. This precipitate was redissolved in Buffer I (20 mM sodium phosphate buffer containing either 250 mM sodium chloride or 500 mM sodium chloride, pH 7). The chelating gel was charged with metal ions (copper, nickel or zinc) then equilibrated with Buffer I. The redissolved cryoprecipitate was applied to a packed column containing the charged, equilibrated gel. After all the material had been applied, the column was washed with Buffer I. The fibrinogen was eluted by washing the column with Buffer II (20 mM sodium phosphate, 0.05 M EDTA buffer containing either 250 mM sodium chloride or 500 mM sodium chloride, pH 7) or Buffer III (20 mM sodium phosphate, 50 mM arginine, 250 mM sodium chloride, pH 7.5) or Buffer IV (20mM sodium phosphate, 200mM arginine, 250mM sodium chloride pH7.0).

The results are shown in Table 4.

Table 4

Gel	Metal ion	Fibrinogen eluted, mg per ml of gel	Factor XIII eluted, u/mg fibrinogen
Toyopearl Chelate AF (Tosoh Biosep)	copper	38.7[a]	0.3
Toyopearl Chelate AF (Tosoh Biosep)	copper	24.1[b]	0.1
Toyopearl Chelate AF (modified spacer arm) (Tosoh Biosep)	copper	35.6[b]	0.08
Fractogel EMD Chelate (Merck)	copper	30.8[a]	0.26
Chelating Sepharose FF (Amersham Biosciences)	copper	16.1[a]	not tested
Toyopearl Chelate AF (Tosoh Biosep)	zinc	23.5[a]	0.22
Fractogel EMD Chelate (Merck)	zinc	31[a]	0.27

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| Chelating Sepharose FF (Amersham Biosciences) | zinc | 11.5[a] | not tested |
| Toyopearl Chelate AF (Tosoh Biosep) | nickel | 23.7[c] | not tested |

[a] Eluted with Buffer II.

[b] Eluted with Buffer III.

[c] Eluted with Buffer IV.

The results show that fibrinogen and factor XIII can be isolated from a fibrinogen-containing solution using immobilised metal ion affinity chromatography (IMAC) with a variety of IMAC base matrix chemistries and metal ions.

Example 12: choice of starting materials

The ability of immobilised metal ion affinity chromatography (IMAC) to purify fibrinogen from different fibrinogen-containing solutions was investigated.

Fibrinogen-containing solutions:

A.   Redissolved Cryoprecipitate obtained from thawed human plasma (prepared according to Example 1).

B.   Redissolved Heparin Precipitate obtained from redissolved cryoprecipitate which had been mixed with heparin (prepared according to Example 2).

C.   Redissolved Cold Precipitate obtained by chilling redissolved cryoprecipitate.

D.   Fibrinogen-containing solution chromatographically depleted of factor VIII, von Willebrand factor (vWF) and fibronectin, obtained from human plasma.

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The cold precipitate used in C was prepared as follows:
cryoprecipitate, prepared as in Example 1, was
redissolved in four times its weight of 50μM calcium
chloride solution at 28°C. pH was adjusted to 6.8 with
5   1M acetic acid and the solution cooled to 10°C. After
mixing for >10 minutes, the precipitate which formed was
collected by centrifugation.

Each solution A-D, containing approximately 1-20 mg
fibrinogen per ml, was incubated with solvent and
10   detergent to inactivate viruses, then applied to a
column of Toyopearl Chelate IMAC resin which had been
charged with copper ions and equilibrated with 20 mM
sodium phosphate, 250 mM sodium chloride pH 7.0. After
loading, the resin was washed with the same buffer.
15   Fibrinogen was eluted by application of 25 mM imidazole
(Buffer X) or 50 mM arginine 20 mM sodium phosphate, 250
mM sodium chloride pH 7.5 (Buffer Y).

The results are shown in Table 5.

20

Table 5

Start Material	Fibrinogen in Start Material, mg/ml	Elution Buffer	Fibrinogen in eluate, mg per ml of resin
A: cryo-precipitate	14.1	X	29.8
C: cold precipitate	21.6	X	29.0
D: fibrinogen solution	0.73	X	38.7
A: cryo-precipitate	11.1	Y	32.5
B: heparin precipitate	14.8	Y	29.9

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C: cold precipitate	20.9	Y	31.4

The results show that IMAC can be used to prepare
fibrinogen from different fibrinogen-containing starting
materials.

Example 13: Formulation of fibrinogen to allow rapid re-
solution after freeze-drying and heat-treatment

Fibrinogen which had been eluted from IMAC resin
according to Examples 9 and 10 and concentrated to
approximately 15 mg/ml in 50 mM Arginine, 20mM phosphate
pH 7.5 (Tables 6 and 8) or 50 mM Arginine, 10 mM
tri-sodium citrate pH 7.5 (Table 7) was formulated with
various added compounds, filled into glass vials (20 ml
per vial) and freeze-dried. On completion of freeze-
drying, the vials were sealed under vacuum, then heat-
treated at 80°C for 72 hours to inactivate viruses. The
vials were then reconstituted with water (5 ml per vial)
at ambient room temperature (18°C-25°C). Re-solution time
was measured, as the time between addition of water and
the time at which a clear homogeneous solution without
residual solid matter was observed.

The results are shown in Tables 6, 7 and 8.

Table 6

Arg mM	Polys %	NaCl mM	Cit mM	Suc %	Recons time, mins	Fib after recons, mg/ml	Factor XIII after recons, u/ml
50	0.5	250	10	1.5	4.5	44.3	<1
50	0.1	250	10	1.5	8.1	49.3	<1
50	0.5	250	0	0	4.1	40.0	<0.5

50	0.5	250	10	1.5	6.5	37.0	<0.5
50	0.5	250	0	1.5	5.8	33.1	<0.5
50	0.25	250	0	1.5	4.9	35.4	<0.5
50	0.1	250	0	1.5	3.9	39.9	<0.5
50	0.5	230	0	1.5	4.5	36.6	<0.5
50	0.5	200	0	1.5	3.6	37.5	<0.5
50	0.5	180	0	1.5	4.9	35.0	<0.5
50	0.5	150	0	1.5	4.2	37.9	<0.5
50	0.5	130	0	1.5	5.7	36.8	<0.5
50	0.5	100	0	1.5	7.1	36.9	<0.5
50	0.5	80	0	1.5	7.8	36.9	<0.5
50	0.5	50	0	1.5	12.2	41.3	1.2
50	0.5	50	10	1.5	8.6	43.3	1.3
50	0.5	50	0	2.5	7.6	40.7	1.2

Table 7

Arg mM	Polys %	NaCl mM	Phos mM	Suc %	Recons time, mins	Fib. after recons, mg/ml	Factor XIII after recons, u/ml
50	0.5	250			6.3	63.7	<1.5
50	0.5	50		1.5	4.5	67	2.4
50	0.5	50	20	1.5	2.1	63	1.9
50	0.25	50		1.5	1.4	71.7	1.8
50	0.1	50		1.5	1.6	61.9	1.8
50	0.25	50	20	1.5	2.0	62.1	1.8
50	0.1	50	20	1.5	2.6	70.8	1.7
50	0.5	50		1	4.3	70.1	2.0
50	0.5	50		0.75	2.7	69.1	2.0
50	0.5	50		0.5	2.6	73.3	1.7
50	0.25	50		0.75	3.0	71.7	1.8
100	0.1	50		1.5	8.8	54.8	1.3

| 50 | 0.1 | 250 | | | 7.2 | 53.7 | <1 |
| 120 | 0.1 | 50 | | 1.5 | 5.7 | 55.4 | <1 |

Table 8

Arg mM	Poly %	NaCl mM	Cit mM	Carbo %	Recons time, mins	Fib after recons, mg/ml	Factor XIII after recons, u/ml
50	0.5	50	0	sucrose 1.5%	12.2	41.3	1.2
50	0.5	50	0	trehalose 1.5%	14.5	41.1	1.2
50	0.5	50	0	raffinose 1.5%	13.9	42.5	1.3

Abbreviations used in Tables 6-8:

Arg = arginine

Poly = polysorbate 20

Cit = citrate

Suc = sucrose

Fib = fibrinogen

Recons = reconstitution

Carbo = carbohydrate

Phos = phosphate

The results showed that effective concentration ranges of the formulants were:

Sucrose: 0.5-2.5%

Polysorbate 20: 0.1-0.5%

Sodium Chloride: 50-250mM (50 mM for factor XIII retention)

Arginine: 50-120mM

High concentrations of sodium chloride (250 mM) in

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combination with arginine, polysorbate 20 and a buffer
salt allowed rapid re-constitution of freeze-dried,
heat-treated fibrinogen but factor XIII activity was
lost. Reduction in the sodium chloride content

5   accompanied by the addition of sucrose allowed both
rapid reconstitution and the retention of factor XIII
activity.

Selective combinations of arginine, polysorbate 20,

10   sodium chloride, a suitable buffering salt and a
carbohydrate provided a formulation for fibrinogen and
factor XIII which enabled rapid reconstitution of
freeze-dried, heat-treated product at room temperature,
with retention of fibrinogen and factor XIII activity.

15

Example 14: Ultrafiltration, formulation, freeze drying
and heat treatment

In four independent experiments (A-D), a heparin

20   precipitate was resuspended according to Example 3 and
solvent detergent treated according to Example 4. The
resulting solution was separated by chromatography
according to Example 9 to give an eluate rich in
fibrinogen and factor XIII. This eluate was then

25   concentrated according to Example 10. The concentrate
was formulated to a target of 110 mM arginine, 1.5% w/w
sucrose, 0.1% w/w polysorbate 20, 50 mM NaCl, 10 mM
trisodium citrate and sterile filtered to 0.2 μm
according to Example 7. The filtered concentrate was

30   then aseptically filled, freeze-dried and heat-treated
according to Example 8 to give a double viral
inactivated product.

The eluates, filtered concentrates and products were

35   assayed for clottable protein and factor XIII activity,
and the results are shown in Table 9.

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Table 9

Fraction	property	A	B	C	D
eluate	Clottable protein (mg/mL)	5.8	5.6	4.4	4.52
	factor XIII activity (U/mL)	0.19	0.41	0.18	0.18
filtered concen- trate	Clottable protein (mg/mL)	21.2	20.4	22.1	18.2
	factor XIII activity (U/mL)	0.61	1.43	0.68	0.65
product	Clottable protein (mg/mL)	63.3	45.9	48.8	46.3
	factor XIII activity (U/mL)	1.19	1.64	1.25	1.17
	reconstitution time (min)	9.05	5.35	8.72	11.22

The results show that the fibrinogen can be concentrated
to approximately 20 mg/mL using ultrafiltration. They
also show that the formulation conditions give a product
with a concentration of factor XIII of > 1 U/mL and
reconstitution times << 15 min.

Example 15: The effect of Elution Buffer Concentration
and pH on elution of fibrinogen and copper from a
chelate resin.

Cryoprecipitate was redissolved in buffer 1 (20 mM
phosphate buffer containing 0.25 M NaCl pH6.0) and
solvent detergent treated as Example 4. Chelate resin
(Toyopearl AF Chelate 650 (M)) was charged with copper
ions, pre-washed with the appropriate elution buffer and
then equilibrated with buffer 1. This material was
loaded onto the packed, copper charged resin. After all

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the material had been applied, the column was washed
with fifteen to twenty volumes of buffer 1. The column
was then washed with five to seven bed volumes of buffer
2 (20 mM phosphate buffer containing 0.25 M NaCl pH7.0).
The column was eluted with a buffer containing 20 mM
phosphate, 0.25 M NaCl and arginine at a range of
concentrations and pH.

The results are shown in Table 10.

Table 10

Arginine Concentration in elution buffer	elution Buffer pH	Concentration of Eluted Fibrinogen mg/ml	mgs of Fibrinogen Eluted /ml of Resin	Concentration of Eluted Copper mg/L	μg Copper /mg of Eluted fibrinogen
200	7	4.5	32.3	8	1.8
100	7	4.8	35.7	4.6	0.95
50	7	2.5	17.5	2	0.8
50	6.0	1.9	17.6	1.4	0.74
50	7.5	4.4	33.3	2	0.46
50	8.0	3.96	35.3	2.5	0.63

The results showed that a reduction in the concentration
of arginine in the elution buffer reduced the
concentration of copper in the fibrinogen eluate but had
an adverse effect on the recovery of fibrinogen. An
increase in the pH of the 50 mM arginine elution buffer
to 7.5 resulted in high recovery of fibrinogen and the
levels of copper co-eluted were substantially reduced.

Example 16: Treatment of heparin precipitate

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Frozen heparin precipitate was re-dissolved in 20 mM
sodium phosphate, 500 mM sodium chloride, 10 mM
tri-sodium citrate pH 6.0 at a ratio of 1:5 by weight.
Two re-solution methods were compared:

Method 1. Heparin precipitate was added to the buffer at
ambient temperature. The mixture was then warmed to
40°C and incubated at 40°C for 1 hour.

Method 2. The buffer was pre-warmed to 40°C and the
heparin precipitate was then added at a rate which
maintained a temperature of >36°C. Following addition
of all precipitate, the mixture was then incubated for 1
hr at 40°C.

Three different batches of precipitate were tested. In
each case, re-solution Method 2 resulted in a
significantly higher filter capacity. The final
filtrate weight and fibrinogen concentration was also
consistently higher resulting in improved fibrinogen
yields (see Table 11).

Table 11

Batch	re-solution method	scale of filt-ration[1]	Cuno 05SP filter capacity[2] (kgm^{-2})	Cuno 30LA filter capacity[2] (kgm^{-2})	Sartobran 0.65/0.45 mm filter capacity[2] (kgm^{-2})	Fibr-inogen yield[3] (%)
A	1	Bench	10.66	4.57	3.57	64
A	2	Bench	26.31	26.10	17.51	Not assayed
A	2	Pilot	> 23.57	> 23.72	> 13.96	82
B	1	Bench	4.33	4.57	3.47	29
B	2	Bench	13.40	13.16	11.69	Not assayed
C	1	Bench	9.63	8.73	8.48	58
C	2	Pilot	> 21.66	> 22.92	> 13.96	81

Notes:

1 Bench scale filter area = 0.00173 m^2 ; pilot scale
filter area = 0.3 m^2. The initial applied air pressure
during filtration was 0.25 bar (25,000 Pa) at bench
5   scale and 0.1 - 0.2 bar (10,000-20,000 Pa) at pilot
scale.

2 Filter capacity is calculated as the maximum amount
of re-solubilised material filtered divided by the
filter area. Examples showing capacities "greater than"
10   did not block the filter under experimental conditions

3 The fibrinogen yield is calculated in this case as
the mg of fibrinogen recovered from the filter (=
concentration recovered mgmL^{-1} x mL filtrate collected)
divided by the mg fibrinogen that was in this volume of
15   starting material (= concentration applied mgmL^{-1} x mL
filtrate collected) x 100(%).